

## Composition and Densities of $\beta$ -Lactoglobulin Crystals in Sucrose and Serum Albumin Solutions

The densities and hydration of a number of virus proteins have been determined by sedimentation rates in media of different densities.<sup>2-7</sup> Solutions of sucrose, urea, glycerol<sup>3</sup> and serum albumin<sup>5</sup> with different densities were used in making these measurements. These investigations show that the densities of viruses in solution are much higher in sucrose solutions than in serum albumin solutions.<sup>5,7</sup> It has also been reported<sup>3,5</sup> that the sedimentation rate of a virus in sucrose solutions is not a linear function of the sucrose concentration. Recent measurements,<sup>7,8</sup> however, indicate that the sedimentation rate of a virus in sucrose solutions is a linear function of the density of the solution.

It was thought that a study of the composition and densities of  $\beta$ -lactoglobulin crystals after equilibration in solutions of serum albumin and sucrose would be valuable in interpreting sedimentation data on virus proteins in similar solutions. Adair and Adair<sup>9</sup> have shown that the partial specific volume of a protein is the same in the crystal as in solution. This makes the hypothesis plausible that the hydration of a protein is essentially the same in solution as in the crystal. The results obtained in our study on the densities of  $\beta$ -lactoglobulin crystals in these solutions parallel the densities reported for viruses by sedimentation rates. It was found that sucrose penetrates the protein crystal and that the relation of the concentration of sucrose in the crystal to that of the medium varied with the concentration of sucrose. In contrast to this behavior, the composition of  $\beta$ -lactoglobulin crystals was unchanged when the crystals were suspended in serum albumin solutions, demonstrating that serum albumin did not go into the  $\beta$ -lactoglobulin crystal.

### Materials and Methods

**$\beta$ -Lactoglobulin Crystals.**—Palmer's method,<sup>10</sup> involving the separation of the  $\beta$ -lactoglobulin fraction with ammonium sulfate, was used in making crystalline  $\beta$ -lactoglobulin. After two recrystallizations from dilute sodium chloride solutions, large crystals were made by diluting a

saturated solution of  $\beta$ -lactoglobulin in a 0.1 *N* solution of sodium chloride with an equal volume of water. On standing for several days at 2–4°, large crystals formed. The supernatant liquor was removed with a pipet, and the crystals were washed free of chloride. Toluene was used as a preservative.

The bovine serum albumin was obtained from Armour and Company. The sucrose was a Bureau of Standards preparation. Solutions were made by weight per cent. Densities of sucrose solutions were taken from the "International Critical Tables." The hydration, densities and protein contents of  $\beta$ -lactoglobulin crystals were determined by methods previously described.<sup>11</sup> Large  $\beta$ -lactoglobulin crystals weighing from 5 to 18 mg. were placed in solutions of serum albumin and sucrose contained in a closed vessel, and kept at 2 to 4°. After standing for twenty-four hours, which was sufficient time for equilibrium, the vessels were warmed to 25° and allowed to stand about one hour; periods of standing at 25° up to 7 hours did not alter the composition. A crystal was removed and carefully blotted with soft filter paper. The crystal was then weighed on a balance sensitive to 0.01 mg. The variation of weight with time was determined, and the weight at the time of blotting obtained by extrapolation. The dry weight was determined by heating to constant weight at 110°. The total water content was considered to be the difference between the extrapolated wet weight and the dry weight. For determination of sucrose and protein contents, the dried crystal was suspended in 2 cc. of boiling water for three minutes, and then the supernatant, which contained sucrose, was removed. This extraction procedure was repeated twice, and the sucrose was determined in the extract by the method of Stiles, Peterson and Fred.<sup>12</sup> The extracted crystal was again dried to constant weight. This weight, considered to be the protein content of the crystal, was in excellent agreement with the value obtained by subtracting the sucrose content from the total dry weight.

For determination of density, the  $\beta$ -lactoglobulin crystal was blotted rapidly and dropped into a tube containing a mixture of bromobenzene and xylene (BBX). Suitable additions of these liquids were made until the crystal was suspended. The density of the flotation liquid was determined. To obtain more precise density values, further determinations were made on a series of flotation liquids in which the densities varied by 0.002. The density results are accurate to  $\pm 0.002$ .

No attempt was made to demonstrate the absence of serum albumin in the  $\beta$ -lactoglobulin crystals after they were suspended in serum albumin solutions. It was considered that the density determinations on such crystals were sufficiently precise to detect the presence of any appreciable change in their composition.

The solubility of  $\beta$ -lactoglobulin in sucrose and serum albumin solutions is of the same order as its solubility in water, being about 0.1%. The presence of 0.1% dissolved  $\beta$ -lactoglobulin is considered to be of negligible influence on the results obtained.

### Results

**$\beta$ -Lactoglobulin Crystals Suspended in Serum Albumin Solutions.**—No difference was detected between the densities of the  $\beta$ -lactoglobulin crystals equilibrated in water and in serum albumin solutions (Table I).

- (11) McMeekin and Warner, *THIS JOURNAL*, 64, 2393 (1942).  
(12) Stiles, Peterson and Fred, *J. Biol. Chem.*, 12, 427 (1927).

(1) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

(2) McBain, *THIS JOURNAL*, 58, 315 (1936).

(3) Smadel, Pickels and Shedlovsky, *J. Expt. Med.*, 68, 607 (1938).

(4) Lauffer and Stanley, *ibid.*, 80, 531 (1944).

(5) Sharp, Taylor, McLean, Beard and Beard, *J. Biol. Chem.*, 159, 29 (1945).

(6) Miller and Price, *Arch. of Biochem.*, 10, 467 (1946).

(7) Schachman and Lauffer, *THIS JOURNAL*, 71, 536 (1949).

(8) Taylor and Lauffer *Abs. of Papers*, p. 18C, 116th A. C. S. Meeting, September, 1949.

(9) Adair and Adair, *Proc. Roy. Soc. (London)*, B120, 422 (1936).

(10) Palmer, *J. Biol. Chem.*, 104, 359 (1934).

**$\beta$ -Lactoglobulin Crystals Suspended in Sucrose Solutions.**—Tables I, II and III show the changes in the composition of  $\beta$ -lactoglobulin crystals suspended in sucrose solutions. Densities determined in bromobenzene and xylene mixtures (BBX) are in good agreement with calculated densities. Densities were calculated by means of the formula  $\bar{v} = v_p X_p + v_{H_2O} X_{H_2O} + v_s X_{\text{sucrose}}$ , where  $\bar{v}$  = specific volume of the crystal,  $v_p$  = the partial specific volume of protein (0.753) and  $X_p$  = the weight fraction of protein;  $v_{H_2O}$  = the partial specific volume of water (1.0) and  $X_{H_2O}$  the weight fraction of water;  $v_s$  = the partial specific volume of sucrose (ranging from 0.616 to 0.626, depending

on the concentration) and  $X_{\text{sucrose}}$  = the weight fraction of sucrose.

As shown in Fig. 1, the density of a  $\beta$ -lactoglobulin crystal suspended in sucrose solutions is a linear function of the density of the suspending medium, but  $\beta$ -lactoglobulin crystals suspended in serum albumin solutions are not influenced by the serum albumin.

TABLE I  
COMPOSITION AND DENSITIES OF  $\beta$ -LACTOGLOBULIN CRYSTALS EQUILIBRATED IN SUCROSE AND SERUM ALBUMIN SOLUTIONS AT pH 5.2 AND 25°

Suspending medium	Concn., wt. %	Water content of $\beta$ -lactoglobulin crystal, %	Albu- min or sucrose in crystal water, wt. %	Density Calcd.	Density Dtd. in BBX mix- tures
Water	100	46.5	...	1.152	1.153
Serum albumin	9.15	47.0	0	1.152	1.153
Serum albumin	18.3	45.0	0	1.152	1.153
Sucrose	6.45	43.9*	2.49	1.164	1.163
Sucrose	12.9	42.7*	6.00	1.175	1.172
Sucrose	24.2	39.8*	11.70	1.190	1.189
Sucrose	34.7	38.5*	20.73	1.209	1.210
Sucrose	53.7	33.7*	35.3	1.243	1.255

\* Calculated from the weight of water and protein, omitting the weight of sucrose.

TABLE II  
COMPOSITION OF  $\beta$ -LACTOGLOBULIN EXPRESSED AS VOLUME FRACTION\*

Suspending medium sucrose, wt. %	Water	Volume fraction of Protein	Sucrose	Specific volume of crystal
0	0.465	0.403	0	0.868
6.45	.434	.418	.0068	.859
12.9	.416	.419	.0164	.851
24.2	.378	.431	.0312	.840
34.7	.350	.420	.0570	.827
53.7	.285	.422	.0970	.804

\* Volume fraction, as used here and in Figs. 3 and 4, is defined as the contribution of the component to the specific volume of the crystal.

TABLE III  
EFFECT OF SUCROSE ON THE WATER CONTENTS AND VOLUME OF  $\beta$ -LACTOGLOBULIN CRYSTALS

Concn. sucrose, wt. %	G. water/g. protein	Loss of water, ml./g. protein	Vol. sucrose in crystal, ml./g. protein	Contraction of crystal in volume, ml./g. protein
0	0.873	...	...	...
6.45	.783	0.090	0.012	0.078
12.9	.745	.128	.029	.099
24.2	.661	.212	.054	.158
34.7	.626	.247	.092	.155
53.7	.508	.365	.171	.194

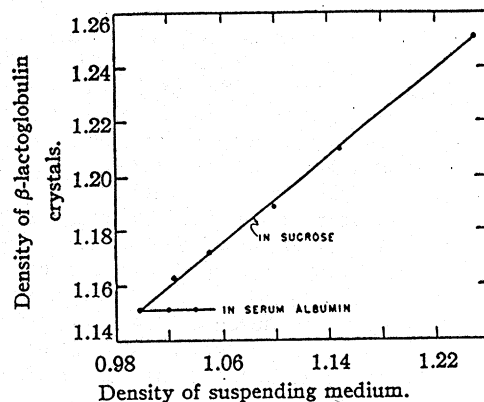
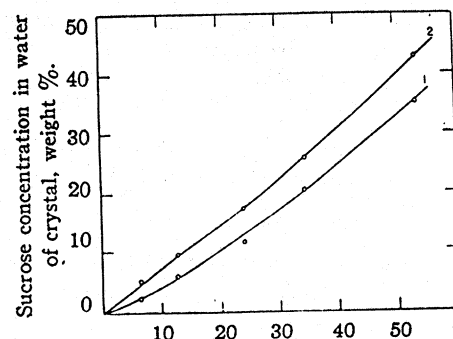


Fig. 1.—Densities of  $\beta$ -lactoglobulin crystals equilibrated in sucrose and serum albumin solutions.

The water contents of the crystals were determined by difference between the wet weight and the dry weight of the crystal rather than calculated from the densities. The weight per cent. of sucrose in the water of the crystal was calculated from the sucrose and water contents. Figure 2, curve 1, shows the relationship of the sucrose content in the water of the crystal to that of the suspending medium. The ratio of the concentration of sucrose in the water of the crystal to that in the suspending medium is not a constant but ranges from about 38% in the lowest to about 65% in the highest sucrose concentration. In contrast to curve 1, Fig. 2, which represents the analytically determined sucrose concentration, curve 2 repre-



Sucrose concentration in suspending medium, weight %.

Fig. 2.—Effect of concentration of sucrose in the suspending medium on the sucrose content of the  $\beta$ -lactoglobulin crystal. Curve 1 represents data obtained by analysis for sugar, protein and water. Curve 2 represents concentration of sucrose in the crystal calculated by the equation of Perutz from data on densities.

sents the concentration of sucrose in the water of the crystal, as calculated from densities with the equation of Perutz<sup>13</sup> for calculating the specific volume of the liquid of crystallization

$$v = (V - nMv_p)/(VD - nM)$$

where  $v$  = specific volume of the liquid of crystallization,  $V$  = unit cell volume in  $\text{\AA}^3$ ,  $n$  = number of  $\beta$ -lactoglobulin molecules per unit cell,  $M$  = molecular weight of  $\beta$ -lactoglobulin in  $10^{-24}$  g.,  $D$  = crystal density, and  $v_p$  = partial specific volume of the protein. The values for the unit cell volume, molecular weight, and number of molecules per unit cell as computed by Senti and Warner<sup>14</sup> for  $\beta$ -lactoglobulin were used in these calculations. The concentration of sucrose corresponding to a given specific volume was obtained by interpolation from a standard sucrose density concentration curve. The values obtained by this method of calculating the concentration of sucrose in the water of the  $\beta$ -lactoglobulin crystal (curve 2, Fig. 2) are considerably greater than those obtained by direct analysis. Moreover, the calculated ratio of the concentration of sucrose in the crystal to that in the suspending liquid, about 75%, is largely independent of the concentration of sucrose in the suspending liquid. To apply the equation of Perutz in determining the specific volume of the liquid of crystallization, it is necessary to know the volume of the unit cell. As shown in Table III, the loss of water by  $\beta$ -lactoglobulin crystals is greater than the volume of the sucrose in the crystals; consequently the unit cell volume decreases proportionally to the total loss in volume. If the unit cell changes in volume in the sucrose solution, the basis for the calculation of curve 2 of Fig. 2 is erroneous, since the calculation involves use of a constant volume for the unit cell of the  $\beta$ -lactoglobulin crystal.

Table II shows the composition of  $\beta$ -lactoglobulin crystals suspended in sucrose solutions

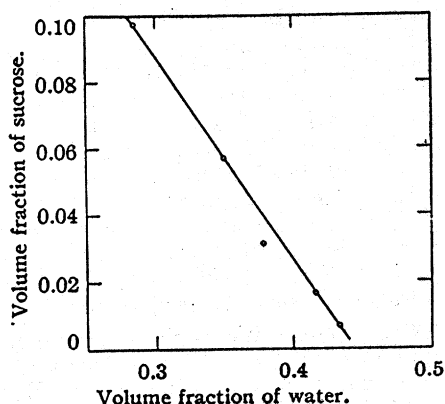


Fig. 3.—Relationship of the volume fraction of water to that of sucrose of  $\beta$ -lactoglobulin crystals in sucrose solutions.

(13) Perutz, *Trans. Faraday Soc.*, **XLII**, 187 (1946).

(14) Senti and Warner, *This Journal*, **70**, 3318 (1948).

expressed as volume fraction. In making these calculations, the specific volume of water was assumed to be 1.0; that of sucrose from 0.616 to 0.626 and that of protein 0.753. The volume fraction of protein changes only slightly with changes in concentration of sucrose, but the volume fraction of sucrose increases and the volume fraction of water decreases in a reciprocal manner. This inverse linear relation between the volume fraction of water and sucrose, shown graphically in Fig. 3, indicates that the factors involved in the distribution of sucrose and water in the crystal are directly related. The slope of the line of  $-0.58$  indicates that approximately 1 g. of sucrose displaces 1 g. of water. This interpretation does not include the shrinkage in the volume of the crystal (Table III). Figure 4 illustrates the composition of the crystal.

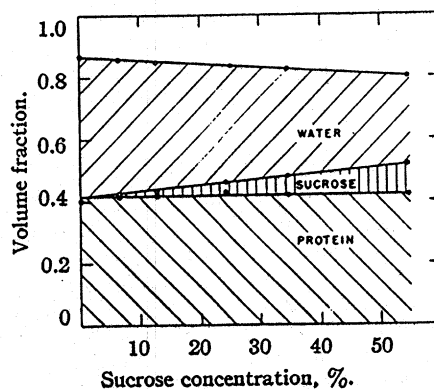


Fig. 4.—Volume fractions of sucrose, protein, and water in crystals of  $\beta$ -lactoglobulin expressed as a function of concentration of sucrose in the suspending medium.

The composition of  $\beta$ -lactoglobulin crystals suspended in sucrose solutions (Table III) shows the contraction in volume per gram of protein, indicating a progressive decrease in volume of the unit cell with increasing concentrations of sucrose. The loss in water per gram of protein of the  $\beta$ -lactoglobulin crystal is directly proportional to the osmotic pressure<sup>15</sup> of the difference in concentration of sucrose in the water of the crystal and in the suspending medium. This relationship is illustrated in Fig. 5.

## Discussion

The density of  $\beta$ -lactoglobulin crystals suspended in serum albumin and sucrose solutions is similar to the density that Schachman and Lauffer<sup>7</sup> found for tobacco mosaic virus in these solutions by sedimentation rates. They report the density of tobacco mosaic virus in serum albumin by sedimentation rate to be 1.13 g./cc. and in sucrose to be 1.27 g./cc. Our value for the density of  $\beta$ -lactoglobulin crystals equilibrated in serum albumin solutions is 1.15 g./cc. and su-

(15) The values of Fraser and Myrick, *ibid.*, **38**, 1907 (1916).

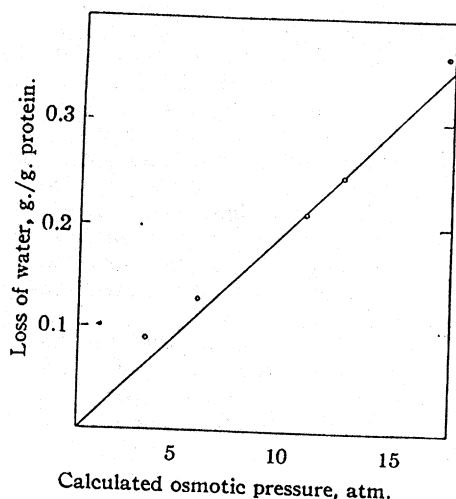


Fig. 5.—Relationship between loss of water by  $\beta$ -lactoglobulin crystals and the osmotic pressure of the difference in sucrose in the crystal water and that in the suspending medium.

crose is greater than 1.255 g./cc. Although there is no reason to expect tobacco mosaic virus to have the identical density as  $\beta$ -lactoglobulin, the densities should be of the same order of magnitude. However, the parallel changes in density in sucrose and serum albumin solutions are suggestive that the densities of viruses in solution are affected by the same factors that operate on the densities of protein crystals. If this be true, it indicates that hydration of a protein in solution is essentially the same as in the crystal state. A further similarity between the density of  $\beta$ -lactoglobulin crystals in sucrose suspension and the results of Schachman and Lauffer<sup>7</sup> on the density of tobacco mosaic virus, and those of Taylor and Lauffer<sup>8</sup> on the density of Southern Bean mosaic virus, in sucrose is the straight line relationship between the density of the particle and the density of the suspending medium.

The hydration of proteins has frequently been calculated from the apparent non-availability of water as solvent for an added substance. Recently, Perutz<sup>13</sup> reported that hemoglobin crystals contain 0.82 g. of water per gram of protein. Based on density determinations of hemoglobin crystals equilibrated in from 1 to 4 molar ammonium sulfate, he deduced that about 0.3 g. of water per gram of protein is not available as solvent and is the hydration of the protein.

Measurements on the composition of  $\beta$ -lactoglobulin crystals in sucrose solutions are particularly valuable for composition studies because the entire sucrose concentration range can be studied. This is impossible in salt solutions because of solution of the protein crystal. The calculated "non-solvent" water per gram of  $\beta$ -lactoglobulin is plotted against the sucrose concentration in Fig. 6. The values for "non-solvent" water vary with the concentration of

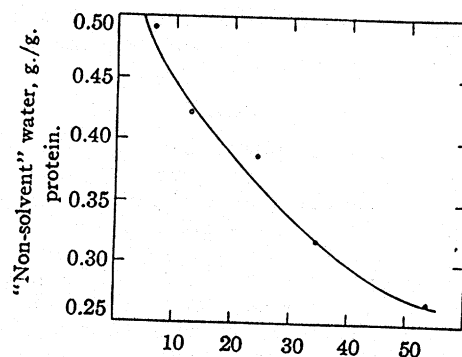


Fig. 6.—Effect of sucrose concentration in the suspending medium on the "non-solvent" water in  $\beta$ -lactoglobulin crystals.

sucrose. At a concentration of 6.4% sucrose, the value is 0.49 g. water/g. protein; at 53.7% sucrose, the value is 0.27 g./of water/g. of protein. These results indicate that the forces involved in determining the concentration of sucrose in the crystal are complicated and do not form a basis for dividing the water of crystallization of this protein in a simple manner. It was of interest to apply the equation of Perutz<sup>13</sup> for "non-solvent" water to the composition of  $\beta$ -lactoglobulin crystals suspended in sucrose, though, as previously indicated, such a calculation is not valid because of probable changes in the volume of the unit cell. This equation, expressed as grams of water per gram of protein, is

$$w = \frac{v - v_m}{v_{H_2O} - v} \left( \frac{VD}{nM} - 1 \right)$$

$v$  = specific volume of crystal liquid;  $v_m$  = specific volume of the suspending medium;  $V$  = volume of the unit cell,  $D$  = density of the crystal;  $n$  = number of molecules in a unit cell; and  $M$  = molecular weight.

The values obtained for hydration by means of this equation ranged from 0.20 to 0.26 g. of water per gram of protein, with the exception of the lowest concentration of sucrose, which gave a value of only 0.12 g. of water per gram of protein.

It has been observed many times that enzyme solutions are more stable in sucrose and glycerol solutions than in water alone. The experiments reported here suggest that the stabilizing effect of sucrose is due to its penetration of the water and dehydration of the enzyme molecule.

**Acknowledgment.**—We wish to acknowledge our indebtedness to Mrs. Janet Pepinsky for the sucrose determinations and to S. R. Hoover and E. F. Mellon for aid in interpreting the data.

### Conclusions

1. The densities of  $\beta$ -lactoglobulin crystals suspended in serum albumin and sucrose solutions are similar to the densities of viruses determined by sedimentation rates in these solutions. This

similarity in behavior is suggestive that the degree of hydration of a protein is essentially the same in solution as in the crystal.

2. The high densities of  $\beta$ -lactoglobulin crystals suspended in sucrose solutions are due to the diffusion of sucrose into the crystal and to a dehydration of the crystal. These effects on the protein crystal indicate that similar effects are present in solutions and that sucrose is not a

suitable medium for density determinations of viruses by sedimentation.

3. The calculated "non-solvent" water of the  $\beta$ -lactoglobulin crystals in sucrose solutions varies widely with the sucrose concentration, making a division of the water crystallization of a protein on this basis of doubtful validity.

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